Photosynthesis II. Electron Transport and Photophosphorylation
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NMR AND ESR STUDIES OF THYLAKOID MEMBRANES

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Abstract

Parallel measurements of water proton relaxation rate ($R_2 = T_2^{-1}$), ESR signal of "free" Mn^{2+} and O_2 evolution activity were used to study the different pools of Mn in thylakoid membranes from pea leaves. The important conclusions of this study [1,2] are reviewed here: (1) Aging of thylakoids at 35°C causes a parallel decrease in O_2 evolution activity, in R_2 and in Mn content, confirming that R_2 is influenced by bound Mn related to O_2 evolution [1]. (2) Addition of 1 to 20 mM $MgCl_2$ causes a decrease in R_2 and an increase in the six-line ESR spectrum for "free" Mn^{2+}, without any effect on O_2 evolution activity; this reflects the presence of a pool of loosely-bound Mn that is non-functional in O_2 evolution [2]. (3) Magnesium ion is known to cause gross structural changes (e.g. grana stacking) in membranes but not in trypsinated membranes; $MgCl_2$ has, however, the same effect upon the R_2 and Mn^{2+} ESR signal of trypsinated as well as untrypsinated membranes. (4) Preparations of isolated light harvesting Chl a/Chl b complex (LHC) contain about a third of the total Mn in the thylakoids, excluding the very loosely bound Mn; this may be the tightly-bound Mn pool [2]. (5) Treatment of thylakoids with NH_2OH increases R_2 by up to nearly two-fold, presumably by the reduction of higher oxidation states of Mn to Mn^{2+}. Also, the progressive release of bound Mn with increasing concentrations of NH_2OH is shown by an increase of the six-line Mn^{2+} ESR signal and by a decrease in R_2 at $[NH_2OH] \geq 1$ mM. (6) Addition of H_2O_2 causes an enhancement of R_2 similar to that by NH_2OH, but without the release of Mn^{2+} [1]. (7) Treatment of thylakoids with tetraphenylboron (TPB) also increases R_2 by two-fold. The titration curve exhibits three sharp endpoints. The first end point occurs at [TPB] ~ 2.5 mM; at this concentration O_2 evolution is completely inhibited [1]. (8) Heat treatment of thylakoids at 35 to 50°C releases Mn^{2+}, as does a pH in the 5 to 4 range. Both high (8 to 9) and low (5 to 4) pH's as well as the heat treatment cause structural changes that increase R_2 [1].

Introduction

Relatively little is known about the molecular mechanism of O_2 evolution of green plants [3]. Some form of membrane bound Mn is involved in this process [4]. Based on the pattern of the amount of O_2 evolution/flash as a function of flash number, B. Kok and coworkers [see ref. 5] have proposed a cyclic model for the accumulation of four oxidizing equivalents by four successive photoacts and the release of one O_2 molecule from two molecules of H_2O; possible changes in Mn were, however, not incorporated in this model. Since manganese ion can take on a number of relatively stable oxidation states, it is a good candidate for the charge accumulator. In an effort to better understand the nature and the role of the manganese in thylakoid membranes we have made parallel NMR and ESR measurements of them in aqueous suspension under a variety of conditions [1,2]. Also, total Mn content was determined by neutron activation and O_2 evolution monitored by a Clark electrode. This work will be briefly reviewed here.

The water proton relaxation rate is known to be sensitive to the amount and oxidation state of Mn present and to the extent and nature of its binding in a complex. Also, changes in binding can affect the correlation times that govern R_2 or change the accessibility of Mn to the aqueous protons. In ESR, "free" aqueous Mn^{2+} gives a distinctive six-line spectrum while other oxidation states and bound Mn^{2+} give at most very broad, weak absorption. The two types of measurements give confirmatory and supplementary evidence of changes in oxidation state and binding of Mn.

In other earlier work two pools of bound Mn have been identified, a loosely-bound pool related to O_2 evolution and a tightly-bound pool to which no role has been assigned. Our experiments provide new information on these pools and on the changes brought about in them by a variety of thermal and chemical treatments.

Effects of Aging on Thylakoid Mn and O_2 Evolution [1]

We varied the Mn pool of thylakoids by aging them at 35°C and studied its effect upon O_2 evolution activity and R_2. Thylakoids were aged in a buffer (at pH 8.3) preequilibrated at 35°C (see ref. 6, for details of the procedure). At specific times aliquots were removed, quenched in buffer at 4°C, centrifuged and the pellets suspended in a buffer (pH 7.4) containing 1 mM EDTA. Thylakoids were recentrifuged and resuspended in the above buffer without EDTA. Aging of thylakoids results in a time-

dependent loss of O_2 evolution, and a decrease in R_2 and the Mn content of membranes as shown in Fig. 1.

After 3 min. at 35°C, the Mn content, as measured by neutron activation analysis, decreased to about 30% (from 0.69 to 0.22 μg Mn/.mg Chl; Fig. 1, open circles). The steady-state saturation rates of O_2 evolution decreased in parallel as a function of the time of aging; 10 min of incubation at 35°C was enough to inhibit almost all O_2 evolution (Fig. 1, open squares). Similarly, aging caused a parallel decrease in R_2 (Fig. 1, filled circles). This experiment establishes a positive correlation between R_2, the Mn content and O_2 evolution. Clearly, R_2 is influenced by bound Mn related to O_2 evolution.

The amount of Mn remaining after 30 min of aging is only about 5% of the initial total, showing that both the tightly (~1/3) and loosely (~2/3) bound pools are affected. Moreover, the two pools appear to be removed concurrently. The O_2 evolution would fall off more rapidly than the Mn content if the loosely-bound Mn were removed first, and this does not appear to be the case in Fig. 1.

Effects of Mg Ion on Thylakoid Mn as Monitored by ESR, NMR and O_2 Evolution [2]

The Mn associated with photosystem II (PSII) in thylakoids is bound heterogeneously [3]. Removal of the loosely-bound Mn (two-thirds of the loosely plus tightly bound Mn) leads to a proportional decrease in O_2 evolution; this is the pool functional in O_2 evolution. The function of the tightly-bound Mn pool (one-third) is obscure. In order to explore further the heterogeneity of the bound Mn, we have treated thylakoids with low concentrations of $MgCl_2$. Fig. 2 (solid circles) shows the effect of increasing concentrations of added $MgCl_2$ on R_2, oxygen evolution and free

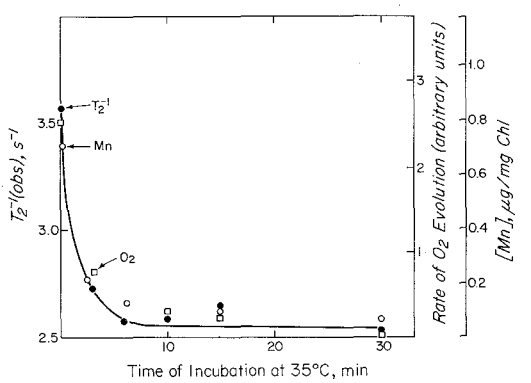

Fig. 1. Effect of time of incubation, at 35°C, of thylakoid membranes on R_2 ($=T_2^{-1}$), the O_2 evolution and Mn content. Obs = observed. (After Khanna et al. [1].)

Mn^{2+} of thylakoid membrane suspension at pH 6.5, partially depleted of cations by washing once with 10 mM NaCl. Qualitatively similar results were obtained on R_2 and the Mn^{2+} ESR signal at pH 7.5 although the Mg^{2+} induced change was somewhat smaller and the concentration needed for 50% change was 6 mM instead of 3.5 mM (at pH 6.5).

In Fig. 2, there is a significant decrease in R_2 from 4.3 to 3.3 s^{-1} as the $MgCl_2$ concentration is increased to 20 mM, above which there is no appreciable further change (at least up to 50 mM). The amplitude of the ESR signal ascribed to free Mn^{2+} increases with increasing concentrations of $MgCl_2$. We attribute the increased ESR signal to "released" Mn^{2+}. The amount of Mn^{2+} so released by treatment with 20-50 mM $MgCl_2$, calculated by comparison with the ESR spectra of aqueous 10 µM $MnCl_2$ solution, was 0.15 µg Mn/ mg Chl. The total Mn content of the untreated membranes was 0.80 µg Mn/ mg Chl.

The Mn^{2+} released by 20-50 mM $MgCl_2$ has little or no effect upon the O_2 evolution (closed squares, Fig. 1). The O_2 evolved per flash is also unaffected. Measurement with a Clark electrode gave a value of one O_2 molecule/2,600 $\pm$ 250 chlorophyll (Chl) molecules, with and without 10 mM $MgCl_2$ at pH 7.5. This value is comparable with the photosynthetic unit size of 2,500 Chl molecules/O_2 reported in Chlorella [7]. The finding of Bose and Arntzen [8] that O_2 evolution is enhanced to a large extent by low $MgCl_2$ concentration appears to be an artifact caused by its effect upon the rate at which thylakoids sediment on the platinum electrode surface [9].

The above results show the presence of a very loosely bound Mn pool (about 20% of the total bound Mn in 10 mM NaCl - washed membranes) that

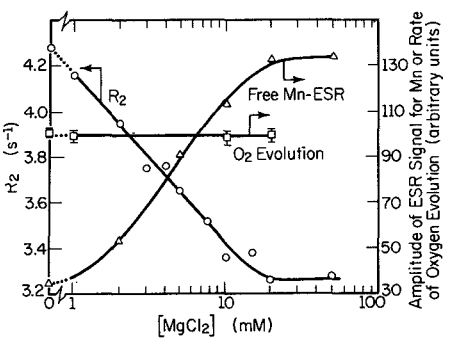

Fig. 2. Effect of $MgCl_2$ in NaCl-washed thylakoid membranes on R_2 ($\equiv T_2^{-1}$), the O_2 evolution and the "free" manganese as estimated by ESR. (After Khanna et al. [2].)

can be displaced by 20-50 mM $MgCl_2$. This pool is clearly not related to O_2 evolution.

Effects of Mg Ion on Trypsin-Treated Thylakoid Membranes [2]

Cations cause major structural and functional changes in thylakoid membranes. It is known that the LHC of chloroplasts is necessary for cation mediated structural changes (grana formation) and energy redistribution between the two pigment systems [10]. The proteolytic enzyme trypsin modifies the LHC at the external surface of the membrane such that the membranes do not exhibit cation induced structural changes [11]. Such structural changes could lead to a change in the environment of the bound Mn, thereby affecting the relaxation of water protons in the hydration sphere of the Mn complex. In order to verify that the decrease in R_2 upon $MgCl_2$ addition (Fig. 2) is influenced by the release of Mn^{2+} and its lower relaxivity and not by the gross structural changes, we performed experiments on trypsin treated thylakoid membranes. The results for R_2 and O_2 evolution showed [2] that R_2 decreases to the same extent when 20 mM $MgCl_2$ is added to the trypsin-treated membranes (~ 12 min treatment) or to the control membranes which were carried through the same incubation without trypsin. Moreover, in both samples the $MgCl_2$ releases Mn^{2+}, as shown by an increase in the 6-line ESR spectrum for free Mn^{2+}. Furthermore, Mg^{2+} ion does not change the rate of O_2 evolution in membranes treated with trypsin for ~ 12 min. Thus, the effect of Mg ion persists in thylakoids that do not undergo gross structural changes; low concentrations of $MgCl_2$ release Mn from a very loosely bound pool not required for O_2 evolution.

Manganese Content and R_2 of the Light Harvesting Complex [2]

The LHC, a pigment protein complex [12], can undergo self association in the presence of cations and bring about cation-mediated grana formation [10]. To further assess the possible effects of structural changes on the observed R_2, we measured R_2 and Mn content of the isolated LHC.

The Mn content of the LHC (0.4 µg Mn/mg Chl) corresponds to 6.6 m moles Mn/mole Chl. For isolated LHC the average mole ratio of Chl/polypeptide (23,000 molecular weight) has been determined to be 13.4 with about 6 polypeptides/LHC, or ~80 Chl/LHC [12]. This gives 0.53 Mn/LHC. Foyer and Hall [13] have also reported the presence of Mn in LHC. Their analysis corresponds to about half as much Mn/LHC as we find. But there are several differences in the materials and procedures. The origin of the Mn in the LHC depends upon the nature of the heterogeneity of Mn binding

in the whole membranes. One possibility is that all of the tightly-bound Mn is associated with and retained by the LHC. The amount of tightly-bound Mn is about a third of the total in whole membranes [3,4], excluding the very loosely bound Mn we report here, i.e. ~ one-third (0.80-0.15) = 0.22 μg Mn/mg Chl. However, at most 60% of the total Chl is in the LHC [14] so the ratio of tightly-bound Mn to Chl in the LHC would be $\geq$ (0.22/0.6) = 0.37 μg Mn/mg Chl. This is consistent with the 0.40 value actually found.

The high content of Mn in the LHC is not due to a nonspecific association of free Mn during the isolation procedure since there is no appreciable loss of Mn upon dialysis of LHC with 1 mM EDTA. Nonetheless, the high content could result from partial removal of Mn not selectively bound to LHC. In any case, it appears that as much as 60-100% of the tightly bound Mn is associated with the LHC. No role has been assigned to the tightly-bound pool of Mn. Possingham et al. [15] showed that Mn deficiency had a large effect on the structure of spinach chloroplasts, leading to progressive disorganization of the lamellar system. It seems likely that the tightly-bound Mn in LHC could play an important role in chloroplasts. A loss of structure could arise directly if Mn acts as an essential structural link in the membrane, or indirectly if Mn participates in some reactions required for the biosynthesis of these membranes.

Effects of Hydroxylamine and H_2O_2 on Thylakoid Membranes [1]

Hydroxylamine is known to serve as an electron donor to PSII (see e.g., 16-18]. At high concentrations, it specifically inhibits the reactions that lead to O_2 evolution. Fig. 3 shows a plot of R_2 as a function of increasing NH_2OH concentration for a fixed time of incubation (30 min). As the concentration of NH_2OH is increased up to 1 mM, R_2 is enhanced (~40%), but at higher concentrations there is a decrease

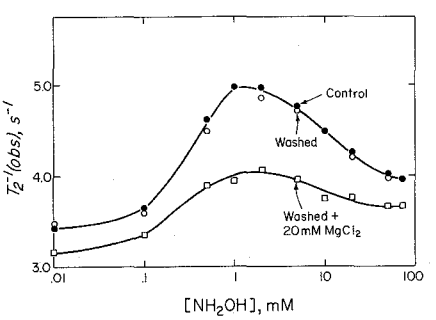

Fig. 3. Effect of NH_2OH in NaCl-washed, washed + 20 mM $MgCl_2$, and control thylakoid membranes on R_2 ($\equiv T_2^{-1}$). (After Khanna et al. [1].)

in R_2 (filled circles). A similar trend is seen for thylakoids washed in 10 mM NaCl (open circles). This indicates that the washing of thylakoids with 10 mM NaCl removes very little or no Mn that undergoes reduction by NH_2OH. However, the addition of 20 mM $MgCl_2$ to the washed thylakoids shows a decreased effect (∿25%) of NH_2OH on the enhancement of R_2 (open squares). ESR spectra show that this decreased enhancement of R_2 by NH_2OH corresponds to the release of Mn^{2+} by $MgCl_2$, probably from the very loosely bound pool of Mn.

R_2 was also measured as a function of time after the addition of NH_2OH for [NH_2OH] from 0.01 to 100 mM. The family of curves so obtained indicates that two processes affect R_2 on a time scale of ∿10 min (at ambient temperatures), the faster one increasing R_2 and the other reversing much of the increase. ESR spectra show that the decrease in R_2 is associated with the progressive appearance of free Mn^{2+}. It seems likely that the enhancement of R_2 is caused by reduction of Mn to Mn^{2+}, but conformational changes might also be important.

Treatment of thylakoids with H_2O_2 (at pH 8.8) also shows an increase in R_2 but without any release of Mn. In the presence of 0.1% H_2O_2, R_2 increases by ∿30% and at higher concentration of H_2O_2 (1.0%) the increase in R_2 is ∿69%. In these experiments, a catalase inhibitor (sodium azide) was used to avoid consumption of H_2O_2 by the endogenous catalase. According to Velthuys and Kok [19] H_2O_2 puts the S states in the more reduced state S_{-1} which is quite stable in the dark. At high pH (8.8), H_2O_2 accelerates the S_1 to S_{-1} reaction and decreases the rate of the reverse reaction (S_{-1} to S_1), thereby increasing the ratio of S_{-1}/S_1. Since S_{-1} is a more reduced state, this is consistent with H_2O_2 increasing R_2 by reducing a more oxidized form of Mn to Mn^{2+}. Unlike NH_2OH, however, H_2O_2 does not increase the ESR spectrum of free Mn^{2+}.

Effect of Tetraphenylboron on Thylakoid Membranes [1]

To further understand the relationship between bound Mn and PSII reactions, we have studied the effects of the reductant TPB on R_2, on PSII reactions, and on ESR of free Mn^{2+}. The plot of R_2 (Fig. 4, filled squares) as a function of added TPB shows three distinct regions of increase related, perhaps, to successive reduction of different pools of ions titrable with TPB. Similar results were reported earlier for the spin-lattice relaxation rate, $R_1 = 1/T_1$ [20]. The increases in R_2 are located at about 2.5, 8 and 25 mM TPB added.

Measurement of O_2 evolution in the presence of TPB (Fig. 4, open

circles) shows complete inhibition of O_2 evolution at 2 mM TPB. This is expected if TPB acts as a competitive electron donor and donates electrons in preference to water [21,22]. A comparison of the rates of O_2 evolution and DCPIP reduction shows that incubation with 1 mM TPB causes 80% inhibition in O_2 evolution whereas electron flow is slowed down by only 26%. A plot of the difference between DCPIP reduction and O_2 evolution (Fig. 4), crosses) represents the net electron flow from TPB to DCPIP. The decline in the net electron flow from TPB at concentrations > 2.5 mM could be due to (a) an inhibition caused by the oxidation product of TPB as suggested by Homann [21] or (b) a reduction of other components of the electron chain thereby preventing electron flow to DCPIP. The latter is lent some support by occurrence of the first break in the titration curve of R_2 versus [TPB] when O_2 evolution is completely inhibited, i.e. at $\gtrsim$ 2 mM.

This leads to the following preliminary interpretation of Fig. 4. At low concentrations ($\leq$ 2 mM) TPB is not a strong enough reducing agent to reduce Mn and affect R_2. At these concentrations TPB donates electrons to PSII in competition with H_2O. At higher concentrations, it not only supplants H_2O completely in the functioning of PSII (no O_2 evolution) but also changes PSII itself, presumably by reducing Mn to Mn^{2+}, or otherwise causing R_2 to increase. The occurence of three breaks in the titration curve could reflect the heterogeneity of the Mn present or different mechanisms by which TPB affects R_2. In this connection, however, the ESR experiments show that TPB does not release Mn^{2+} from the membranes. Further work is needed to establish why there are three end points.

Heating and pH Effects [23]

Incubation of thylakoid membranes for 5 min at 35 to 50°C was found to release Mn^{2+} (per ESR spectra) and to increase R_2, both by amounts increasing rapidly with the temperature. Release of Mn^{2+} decreases R_2

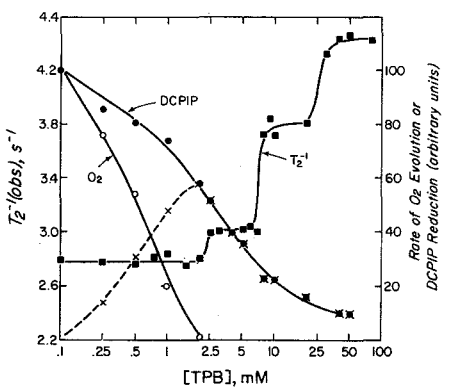

Fig. 4. Effect of tetraphenylboron (TPB) on R_2 ($\equiv T_2^{-1}$), O_2 evolution and dichlorophenolindophenol (DCPIP) reduction in thylakoid membranes. The crosses indicate the difference between DCPIP reduction and O_2 evolution. (After Khanna et al. [1].)

so the heating must produce other changes that increase R_2 enough to more than compensate for the release. These might be structural changes that increase the accessibility and/or correlation times of the bound Mn or, for that matter, of other paramagnetic species such as Cu^{2+} of plastocyanin. This view is supported by the results of fixing the thylakoid membranes with glutaraldehyde before heat treatment and by incubation of other samples with 60 mN KCN after heat treatment. The former cuts the heat-induced increase in R_2 in half while the latter eliminates it.

Extreme pH's also affect R_2 and release Mn^{2+}. The Mn^{2+} release occurs with decreasing pH starting at about 7 and increasing sharply up to 4, our lower limit. On the other hand R_2 increases linearly with increasing pH starting at 7 and extending up to 9, our upper limit. This increase in R_2 is caused probably by structural changes, as in the case of heating, but less extensive. It is likely that such structural changes occur at low as well as high pH; otherwise R_2 would decrease because of the Mn^{2+} release, instead of remaining almost constant.

Concluding Remarks

From our results [1,2,23] it appears that the proton transverse relaxation rate R_2 in aqueous suspensions of thylakoid membranes is influenced by both the functional and non-functional pools of Mn. These include (a) a very loosely bound pool unrelated to O_2 evolution; (b) a loosely-bound pool related to O_2 evolution; and (c) a tightly-bound pool perhaps associated with the LHC. Furthermore, R_2 is sensitive to the oxidation state and environment of the Mn present. It is enhanced by reducing agents which convert into Mn^{2+} some of the Mn present ordinarily in a higher oxidation state. It is decreased by the release of bound Mn as Mn^{2+}, i.e. Mn^{2+} detectable in ESR spectra. It is increased by structural changes in the membrane that increase the accessibility of the Mn or of other paramagnetic species.

Comment should also be made about the effects of light flashes on R_2 of untreated thylakoids. Wydrzynski *et al.* [24] and Govindjee *et al.* [25] found R_2 after a flash to oscillate with the number of flashes with a period of 4. These observations apparently were not artifacts of the measurements (see Govindjee and T. Wydrzynski, these proceedings). However, our attempts since then to reproduce the oscillations have generally met with negative results.

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